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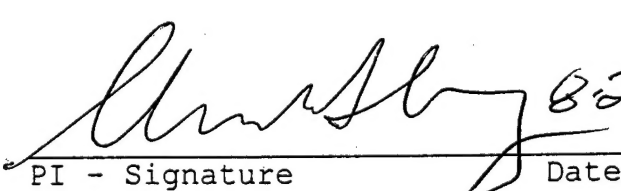
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The p16 Pathway in Breast Cancer and Senescence Control

INTRODUCTION

Chromosomal subregion 9p21 has been shown to undergo hemizygous and homozygous deletion in a variety of tumor types (1-3). Recently, we have reported hemizygous loss to occur frequently in breast cancer (58%) as well (4). Previous analysis of this region had shown it to contain *p16^{INK4a}*, an inhibitor of cyclin dependent kinases, commonly referred to as *p16* (5,6). Nevertheless, when we subjected these same tumors to mutational analysis of *p16*, we observed few tumors with sequence alterations of consequence, thus suggesting *p16* may not be the target of such anomalies in breast cancer (4). However, recent reports have indicated that mutation may not be the primary mechanism of inactivation of the *p16* gene in many tumor types (7-12).

Recently an alternative transcript encoded from the same second and third exons encoding *p16*, and utilizing the same splice site, but with a separate promoter and alternative first exon, now referred to as exon 1 β , has been described (13,14). This alternative transcript is abundant in various tissue types (13,14) and is translated in mouse from an alternative reading frame, resulting in a protein of 19kD with cell cycle arresting capacity, now termed *p19^{ARF}* (15). Thus, the *p16* locus appears to be complex with two overlapping transcripts translated from distinct reading frames, resulting in two polypeptides, *p16^{INK4a}* and *p19^{ARF}*, each able to induce cell cycle arrest. Because the transcripts of these two polypeptides partially overlap, it may be that alterations which affect one, may also affect the other. Therefore, the possibility exists of *p19^{ARF}* being a protein with tumor suppressive function being targeted for inactivation as well. A comprehensive analysis of the aberrations affecting the *p16* and *p19* genes and expression of transcripts *in vivo* should help to clarify these issues.

To help elucidate the role of aberrations affecting these two genes in breast cancer, we have performed a comprehensive analysis of inactivation and expression in a series of primary breast carcinomas. To that end we performed interphase chromosomal fluorescence *in situ* hybridization (IC-FISH) deletion analysis of the *p16* region, methylation analysis of the 5' region of the primary first exon, SSCP analysis of the alternative exon 1 β transcript, and expression of both transcripts (α and β) by semi-quantitative RT-PCR. Taken together with previously determined microsatellite polymorphism LOH analysis of 9p21 and *p16* mutational analysis in these same tumors (4), we believe we have obtained a more complete account of *p16^{INK4a}* and putative *p19^{ARF}* involvement in breast tumorigenesis.

These results have been reported in a recent publication Brenner et al. (Clinical Cancer Res. 2:1993-1998, 1996).

MATERIAL AND METHODS

Tissue Samples, DNA Extraction, and RNA Extraction. Normal and tumor breast samples were obtained from the Cooperative Human Tissue Network. All samples were invasive ductal carcinomas with the exception of cases 24 (ductal carcinoma *in situ*) and 32 (mucinous adenocarcinoma). Samples were snap frozen with liquid nitrogen less than 1 hour after surgery. Regions dense with tumor cells were identified by visual inspection and comparison to H&E slides as necessary. Total genomic DNA was isolated using phenol:chloroform:isoamyl (25:24:1) in Phase Lock Gel tubes (5 Prime \rightarrow 3 Prime, Boulder,

CO), according to standard protocol, and precipitated with 2.5 volumes ethanol. Total RNA was isolated using an RNEasy Total RNA kit (Qiagen, Chatsworth, CA) as per manufacturers instruction.

IC-FISH. Probe pHUR98, a variant satellite 3 sequence which specifically hybridizes to the heterochromatic region of chromosome 9, were used to assess chromosome 9 copy number. p16cos is a contig of eight cosmids encompassing a 250 kilobase region around *p16*, obtained by screening a flow sorted chromosome 9 library (10) and used to determine *p16* copy number. Probes were amplified and labeled by sequence independent amplification (SIA) (16), and either biotin-11-dUTP (Enzo Diagnostics) labeled (centromeric probe pHUR98), or digoxigenin-11-dUTP (Boehringer Mannheim) labeled (*p16* cos contig). Dual color chromosomal FISH was performed as described previously (10). Biotinylated probes were detected with TexasRed-Avidin (Vector Laboratories) and digoxigenin labeled probes detected with FITC conjugated antibodies (Boehringer Mannheim). Gray scale images corresponding to each fluorochrome were captured from tumor cell interphases selected at random, using a Photometrics (Tucson, AZ) CCD cooled camera. Pseudocolor composite images were analyzed using Oncor Image software (Gaithersburg, MD). A total of 150 intact tumor cells were analyzed by three independent observers. Cells with no visible p16cos signal were interpreted as nullizygous for p16 and labeled as total deletions while cells displaying a single copy of the p16 region as well as cells with relative deletion (fewer copies of the p16 region than centromeric pHUR89) were labeled as partial deletions. In order to avoid overinterpretation of incomplete hybridization, based upon analysis of normal human lymph nodes, tumor cell populations were not included in scoring of deletions unless present at $\geq 10\%$.

Southern Hybridization. Methylation analysis was performed as previously described (7,17). Briefly, 10 μ g of genomic DNA were digested with a flanking site enzyme (either Eco RI or Hind III) and a methylation sensitive endonuclease (Sac II, Sma I, and Eag I), ethanol precipitated, resuspended, and resolved in 1% agarose gel overnight. DNA was transferred to a Zeta-Probe nylon membrane (BioRad, Richmond, CA) and hybridized with a 340 bp or 280 bp α -³²P dCTP random prime labeled PCR fragment including exon 1 α , as described (7,17). Autoradiographs were obtained following 2-4 days of exposure.

RT-PCR. Five μ g of total RNA were used for first strand cDNA synthesis with Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) as per manufacturers instructions. Following reverse transcription, each sample was subjected to analysis of GAPDH levels as control of mRNA quantity by PCR amplification using GAPDH Positive Control Primer Set (Stratagene, La Jolla, CA). RT-PCR of both *p16* alpha and beta transcripts was performed as described previously (13). An initial experiment was performed to validate the quantitative nature of the RT-PCR, as previously reported (13), and found to concur (data not shown). Mean normal expression was obtained by analysis of four normal reduction mammoplasty breast samples. Signal intensities in all cases were analyzed and quantified with a Molecular Dynamics Phosphorimager. A relative value of 1.0 was assigned to the ratios of alpha:GAPDH and beta:GAPDH based on normal mean, and subsequent samples normalized accordingly. All experiments were performed in triplicate and standard deviations calculated.

SSCP. Amplification of beta transcripts was performed as described above. RT-PCR products were diluted 2:1 in denaturing loading buffer (95% formamide, 10 mM NaOH, 0.1% xylene cyanol) and resolved in 6% polyacrylamide using two conditions: 10% glycerol, 1XTBE at 16W for 16 hours; 5% glycerol, 0.5XTBE at 8W for 8 hours.

RESULTS AND DISCUSSION

p16^{INK4a} Locus Copy Number. The 9p21 chromosomal subregion has been shown to undergo loss of heterozygosity in a variety of neoplasias (1,18,19). While the *p16^{INK4a}* gene is known to be located within this region, mutational analysis of *p16^{INK4a}* gene in breast tumors revealed infrequent mutations (<5%; ref 4). In order to determine if these tumors may have alternatively incurred homozygous deletion of the *p16* region, and to what extent, we performed dual color interphase chromosomal fluorescence *in situ* hybridization using the 250Kb *p16cos* cosmid contig and a centromeric probe for chromosome 9 copy number. We observed total or partial deletion of the *p16* chromosomal region in 61% (11 of 18) of breast tumors (Table 1). This result is comparable to our aforementioned analysis of the same tumor set by PCR based microsatellite length polymorphism analysis (4). Specifically, three cases (17% of total) displayed tumor cell subpopulations with total deletion of the *p16cos* region. Subsequent analysis by RT-PCR showed low to absent expression in both of the two cases for which sufficient material was available. However, while partial deletion was more common (11 cases) than total deletion, subsequent expression analysis did not show a strong association between partial deletion and loss of expression (Table 1). Tumor cell populations displaying hyperdiploidy of chromosome 9 were observed in eight (45%) of the tumors analyzed. No tumors showed evidence of significant chromosome 9 monosomy.

To our knowledge, this is the first report of homozygous deletion of the *p16* region in primary breast carcinomas through the use of *in situ* hybridization. Although two previous studies have been conducted on breast carcinomas by Southern analysis, cumulatively no homozygous deletions were reported of the 21 breast tumors analyzed (9,20). Since breast cancer samples may also contain a significant portion of normal non-neoplastic stromal or epithelial cells, or heterogeneous tumor cell populations, it may be that Southern analysis is not of sufficient sensitivity for determinations of homozygous loss. However, another study based on microsatellite analysis reported homozygous loss at a frequency comparable to that reported here (21).

Hypermethylation of p16^{INK4a} Exon 1 Alpha. Exon 1 of the *p16^{INK4a}* gene contains a documented CpG island which has been shown to be unmethylated in normal tissue and hypermethylated in certain tumor types at varying incidence (7,8,9). In order to establish the methylation status of the 5' region, total genomic DNA was digested with a combination of a flanking site endonuclease and a methylation sensitive endonuclease, as previously described (9). Twenty three tumors were analyzed, of which patterns consistent with partial or total methylation were observed in four (17%; Table 1; Figure 2). Two tumors (T30 and T41) showed methylation with multiple restriction enzymes (SacII and SmaI), while two others (T16 and T44) revealed methylation with only one enzyme (EagI and SacII, respectively). Of those tumors showing methylation, three (T16,30,41) displayed patterns consistent with methylation of all possible endonuclease sites in that region, while one displayed methylation of a single site (T44). The remaining 19 tumors revealed no pattern consistent with hypermethylation. These results are consistent with a previous report of methylation in primary tumors of the breast, although the frequency observed here (17%) is somewhat lower than the frequency previously reported (31%) by Herman et. al. (9).

Expression of p16^{INK4a} Alpha and Beta Transcripts. As previously suggested by Stone et al., the similarity in size and sequence of the α and β transcripts may have complicated previous efforts to measure *p16* RNA levels by Northern blot (13) in different neoplasias. Only an analysis of transcripts using the unique sequences of exon 1 α would be able to assess the true levels of *p16* expression. Additionally, since inactivating events that

target *p16* may also affect the alternative beta transcript, and since we know the alternative beta transcript to encode p19^{ARF} and have growth suppressive effects in murine cells *in vitro*, it would be advantageous to evaluate both *p16* and alternative beta transcript expression independent of one another.

Expression level of *p16* alpha and beta transcripts in twenty three tumors was determined by RT-PCR analysis. Expression levels of each transcript were subsequently compared to mean normal expression of a panel of four normal breast samples. Expression of both the *p16* primary alpha and alternative beta transcripts in breast tumors was varied (Table 1, Figures 3A and 3B). Six of twenty three (26%) showed expression of *p16*^{INK4a} at levels less than 10% of normal mean, while another 5 (22%) revealed levels of expression from 10% to 30% of normal mean (i.e. greater than 70% reduction in normal expression; see Figure 3). Loss of expression in many of these tumors can be accounted for by either hypermethylation (T16, T30, and T41) or homozygous deletion (T6 and T28). However, inactivation by either of these mechanisms was not observed in some cases in which loss of expression was observed, indicating that other modes of inactivation could be operative. Moreover, a previous report of *p16*^{INK4a} expression by immunohistochemistry suggested loss of expression in as much as 65% of breast tumors (22), indicating that inactivating events might be possible at a post-transcriptional stage as well. We also observed that two additional carcinomas, T2 and T54, displayed what appears to be overexpression of *p16*^{INK4a}, concomitant with beta overexpression. Previous analyses in numerous lines has indicated that the overexpression of *p16* can be associated with Retinoblastoma protein (pRB) inactivation (23). However, no precedent of this association has been described *in vivo*. Nonetheless, such overexpression may be deemed aberrant.

Analysis of beta transcripts showed great variability in expression, with apparent overexpression to be as prevalent as lack of expression. One of the twenty three tumors analyzed revealed undetectable levels of expression (T16). Two additional carcinomas (T6 and T26) showed expression below 30% of the normal mean. Incidentally, the two cases in which the lowest expression of beta was observed (T6 and T16), both displayed alpha loss, and by distinct mechanisms. Loss of expression in tumor T6 appeared to be through homozygous deletion, while loss of expression in tumor T16 appeared to be through methylation and LOH (4). However, methylation of exon 1 alpha only explains loss of expression of the primary alpha transcript. Perhaps in some cases, such as tumor T16, the methylation of the 5' region of exon 1 alpha is indicative of the hypermethylation of the entire locus, and as such, the 5' region of exon 1 beta also could be hypermethylated. Since the 5' region of exon 1β from -180 bp to +266bp contains a 70% GC content, and a CG:GC ratio of 0.71, thus defining a CpG island, this probability exists. Of additional interest, four tumors (T2, T20, T41, and T54) showed considerably high levels of beta (p19^{ARF}) expression between 3 to 5 fold greater than the normal mean. What level of increased expression can be considered significant, and the possible implications of such overexpression, have yet to be determined.

The possibility of p19^{ARF} being tumor suppressive in function has been previously postulated (13,15). However, this has not yet been shown, and an analysis of the expression of this transcript in neoplastic tissue was not previously reported. Previous attempts to address this issue through sequence analysis of exon 1 beta in other tumor types revealed no mutations (14). In this report, we addressed the issue of possible beta inactivation in breast cancer by performing SSCP analysis of the exon 1 region of beta transcripts in all twenty three tumors for which we obtained expression data, and found no evidence of mutation in any of the tumors (data not shown). While our own previous analysis (4) of exon 2 in breast tumors revealed three mutations of 21 tumors affecting amino acid sequence for

the beta transcript (CGA-GGA, codon 87; GCA-GTA, codon 96; CGC-CAC, codon 161), only one of these mutations was found in a region conserved in both mouse and human, and another was a frequently reported polymorphism. Further, we have now shown that apparent loss of expression of the beta (p19^{ARF}) transcript is primarily observed in those breast tumors in which *p16^{INK4a}* expression is compromised. Taken together, this information indicates that point mutation or loss of expression is not common for the beta transcript, and that there is no evidence to suggest a tumor suppressive role for the beta transcript in breast carcinogenesis. However, it is unclear the reason for, and possible consequences of, the observed overexpression of the beta transcript in some breast tumors. Further experiments are needed to address these issues.

Nevertheless, comprehensive analysis of homozygous and hemizygous deletion, methylation, mutation, and expression suggest that the tumor suppressor *p16^{INK4a}* is cumulatively affected in approximately 40-50% of the breast carcinomas analyzed. This rate of inactivation of *p16^{INK4a}*, and lack of inactivation of the beta transcript, implicate *p16^{INK4a}* involvement in the tumorigenesis of the breast at a rate greater or equal to that previously reported for any other tumor suppressor gene in sporadic breast cancer.

Table 1

Tumor #	Deletion Status % of Cells ^a		Methylation ^b	Alpha (p16) Expression ^c	Beta (p19) Expression ^c
	<u>Total</u>	<u>Partial</u>			
2	-	23	-	↑	↑
4	nd	nd	-	↓↓	N
6	68	11	-	↓↓	↓
8	-	44	-	↓	N
10	-	-	nd	↓	N
11 ^d	-	36	nd	N	N
14	-	11	-	↓↓	N
16	nd	nd	+	↓↓	↓↓
18	-	-	-	↓↓	N
20 ^e	-	28	-	N	↑
22 ^d	-	47	-	↓	N
24 ^d	-	-	-	N	N
26 ^d	nd	nd	-	N	↓
28 ^d	44	15	-	↓↓	N
30 ^d	-	-	+	↓	N
32 ^d	-	-	-	N	N
34 ^d	-	-	-	N	N
36 ^d	-	-	-	N	N
38 ^d	nd	nd	-	N	N
41 ^d	nd	nd	+	↓↓	↑
44 ^d	nd	nd	+/-	N	N
48 ^d	10	50	-	nd	nd
50 ^d	-	23	-	N	N
54	-	23	-	↑	↑

^a Determined by the relative signal of p16 cos to chromosome 9 centromer in IC-FISH analysis. Losses in less than 10% of cells were not considered significant and are not shown.

^b Methylation status determined by Southern analysis as described in Methods, - = no methylation, + = methylation, +/- = partial methylation.

^c Expression determined by RT - PCR analysis as described in Methods, ↓↓ = below 10% of normal mean expression, ↓ = below 30% of normal mean expression, ↑ = greater than 300% of normal mean expression, N = normal expression.

^d wild-type p16 exon 2 (ref 4).

^e polymorphism base 140, exon 2 (ref 4).

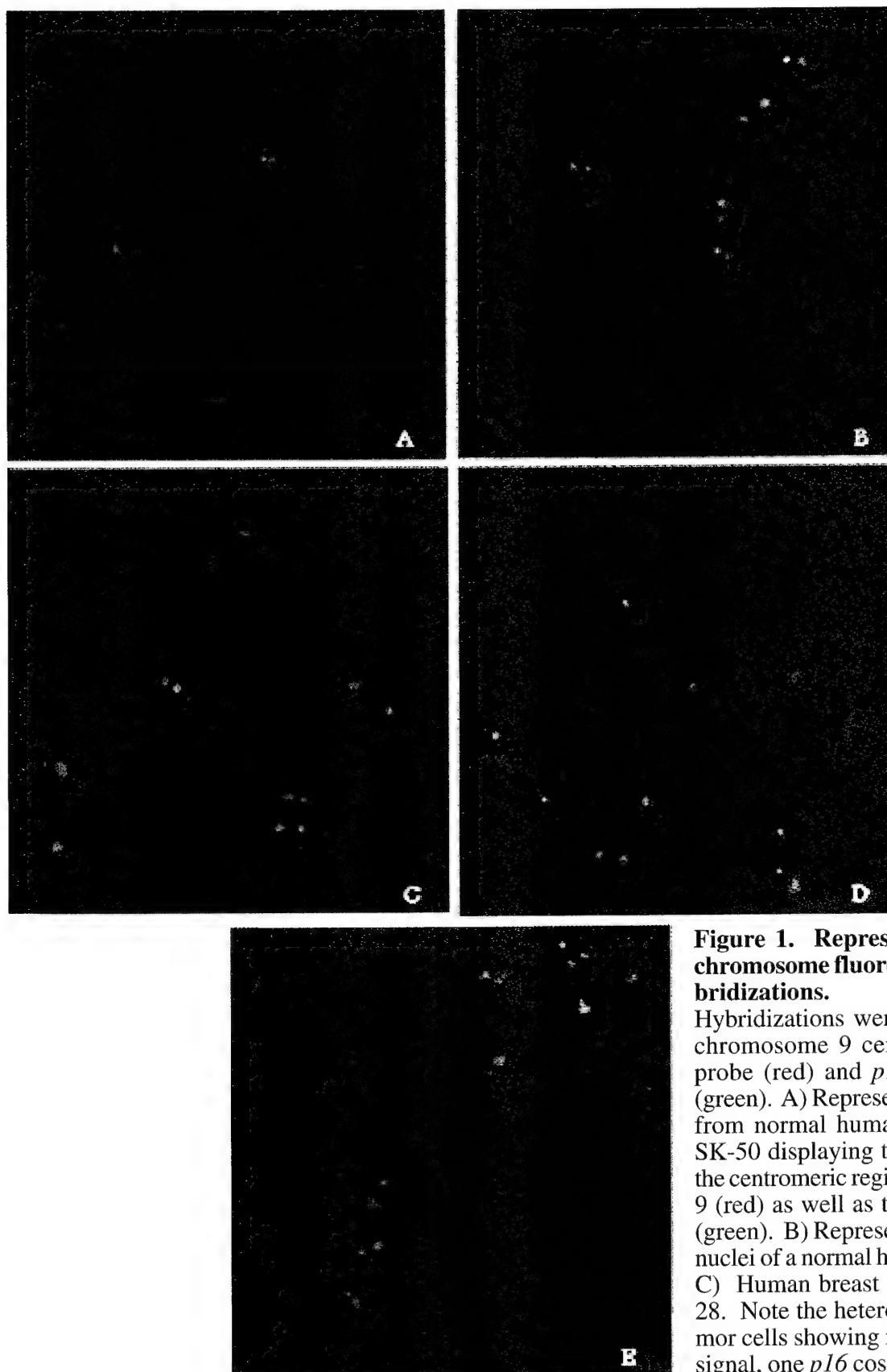


Figure 1. Representative bi-color chromosome fluorescence *in situ* hybridizations.

Hybridizations were performed with chromosome 9 centromere specific probe (red) and *p16* specific probe (green). A) Representative metaphase from normal human fibroblast line SK-50 displaying two alleles of both the centromeric region of chromosome 9 (red) as well as the *p16* cos region (green). B) Representative interphase nuclei of a normal human lymph node. C) Human breast carcinoma sample 28. Note the heterogeneity of the tumor cells showing no *p16* cos (green) signal, one *p16* cos signal, or two *p16* cos signals. D) Human breast carcinoma sample 6. E) Human breast carcinoma sample 22.

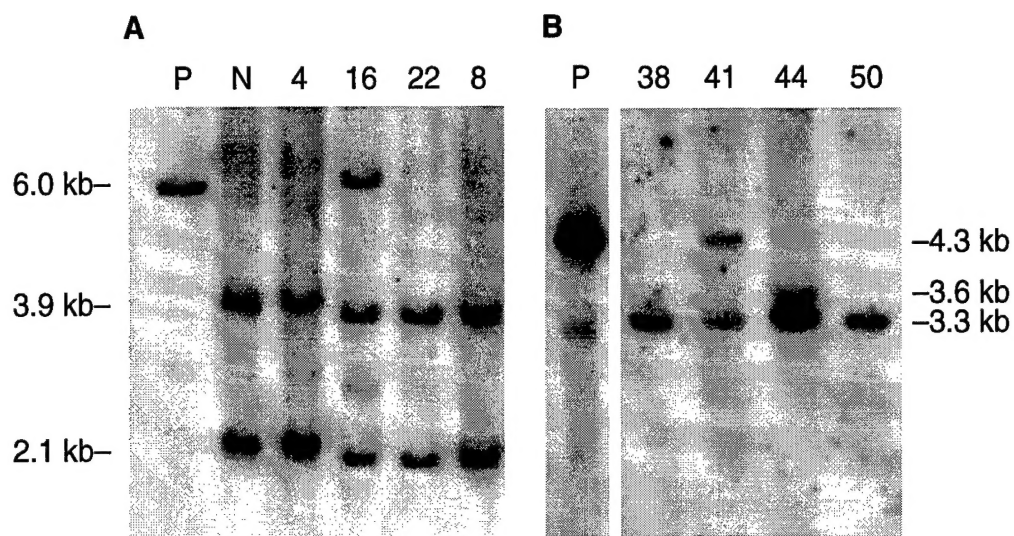


Figure 2. Representative Southern analysis of -p16- exon 1 alpha in primary breast carcinomas.
A. Analysis of methylated line T47D (P, positive control; ref. 9), normal breast DNA (N), and 4 breast carcinomas with Hind III and Eag I reveal methylation of sample 16. B. Analysis of methylated line T47D (P), and 4 breast carcinomas with endonucleases EcoRI and Sma II reveal complete methylation of tumor 41 and partial methylation of tumor 44.

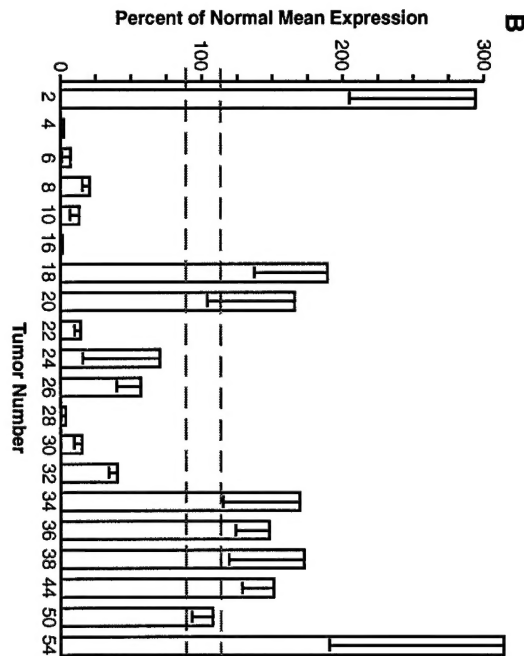
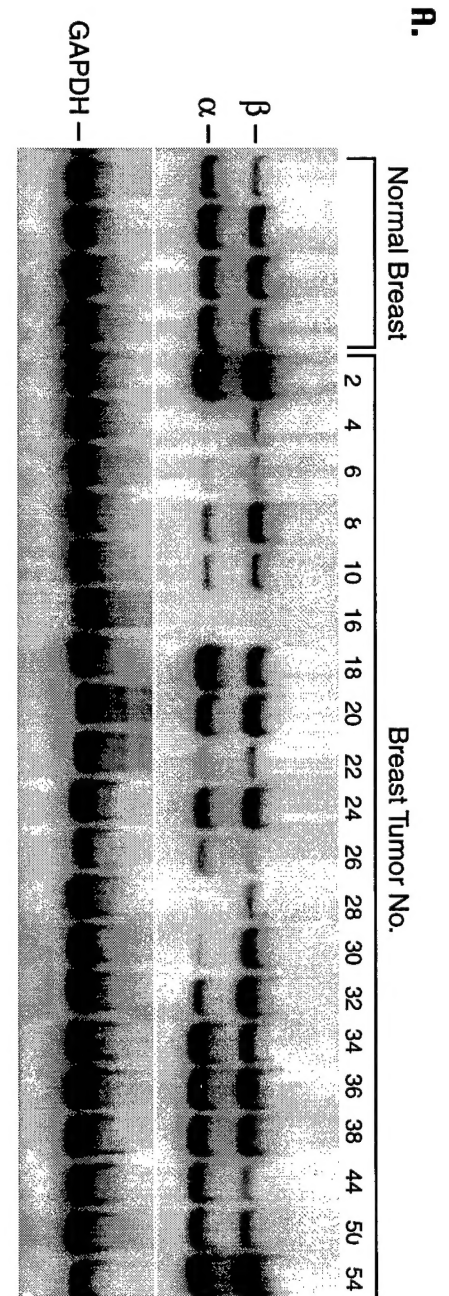


Figure 3. Expression analysis of the alpha (p16INK4a) and beta (p19ARF) transcripts in breast cancer.
A. Autoradiograph of a representative RT-PCR analysis of alpha and beta transcripts in 4 normal breast samples and 20 carcinomas of the breast. B. GAPDH normalized expression levels of the alpha transcript as determined by triplicate RT-PCR analysis in 20 tumors (see text). Dotted line, normal mean SD; bar, sample SD).

CONCLUSIONS

To clearly elucidate the target of aberrations affecting the 9p21 subchromosomal region and approximate frequency in breast cancer, we performed a comprehensive study including *p16* deletion analysis by means of interphase chromosomal fluorescence *in situ* hybridization (IC-FISH), methylation analysis of the first exon encoding *p16^{INK4a}* (exon 1 α), mutational analysis of exon 1 β by single strand conformational polymorphism (SSCP) analysis of *p19^{ARF}* transcripts, and expression of both alpha and beta transcripts by RT-PCR. Homozygous deletion of *p16*, as determined by IC-FISH, was observed in 3 of 18 (17%) tumors analyzed, while *de novo* methylation of exon 1 alpha was observed in an additional 17% (4 of 23). Reduced expression of *p16^{INK4a}* was observed in 11 tumors (48%), including all those in which homozygous deletion or complete methylation was observed. No mutations of exon 1 beta were detected and expression of its transcript was variable with 13% demonstrating decreased expression and 17% demonstrating over-expression. These results further support *p16^{INK4a}* as a target of inactivation in the 9p21 region for breast cancer. We will continue with additional studies as indicated in the original application.

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